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(54) Title: HUMAN GAMMA 3 GABA-A RECEPTOR SUBUNIT AND STABLY CO-TRANSFECTED CELL LINES

(57) Abstract

The present invention relates to the cloning of a novel cDNA sequence encoding the γ_3 receptor subunit of the human $GABA_A$ receptor; to stably co-transfected eukaryotic cell lines capable of expressing a human $GABA_A$ receptor, which receptor comprises at least one α receptor subunit, at least one β receptor subunit and the γ_3 receptor subunit; and to the use of such cell lines in screening for and designing medicaments which act upon the human $GABA_A$ receptor.

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GA	Gabon				

Human gamma 3 GABA-A receptor subunit and stably co-transfected cell lines

This invention concerns the cloning of a novel cDNA sequence encoding a particular subunit of the human $GABA_A$ receptor. In addition, the invention relates to a stable cell line capable of expressing said cDNA and to the use of the cell line in a screening technique for the design and development of subtype-specific medicaments.

Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system. It mediates fast synaptic inhibition by opening the chloride channel intrinsic to the $GABA_A$ receptor. This receptor comprises a multimeric protein of molecular size 230-270 kDa with specific binding sites for a variety of drugs including benzodiazepines, barbiturates and β-carbolines, in addition to sites for the agonist ligand GABA (for reviews see Stephenson, *Biochem. J.*, 1988, 249, 21; Olsen and Tobin, *Faseb J.*, 1990, 4, 1469; and Sieghart, *Trends in Pharmacol. Sci.*, 1989, 10, 407).

Molecular biological studies demonstrate that the receptor is composed of several distinct types of subunit, which are divided into four classes (α, β, γ, and δ) based on their sequence similarities. To date, six types of α (Schofield *et al.*, *Nature (London)*, 1987, 328, 221; Levitan *et al.*, *Nature (London)*, 1988, 335, 76; Ymer *et al.*, *EMBO J.*, 1989, 8, 1665; Pritchett & Seeberg, *J. Neurochem.*, 1990, 54, 802; Luddens *et al.*, *Nature (London)*, 1990, 346, 648; and Khrestchatisky *et al.*, *Neuron*, 1989, 3, 745), three types of β (Ymer *et al.*, *EMBO J.*, 1989, 8, 1665), two types of γ (Ymer *et al.*, *EMBO J.*, 1990, 9, 3261; and Shivers *et al.*, *Neuron*, 1989, 3, 327) and one δ subunit (Shivers *et al.*, *Neuron*, 1989, 3, 327) have been identified.

The differential distribution of many of the subunits has been characterised by *in situ* hybridisation (Sequier *et al.*, *Proc. Natl.

Acad. Sci. USA, 1988, 85, 7815; Malherbe *et al.*, *J. Neurosci.*, 1990, 10, 2330; and Shivers *et al.*, *Neuron*, 1989, 3, 327) and this has permitted it to be speculated which subunits, by their co-localisation, could theoretically exist in the same receptor complex.

Various combinations of subunits have been co-transfected into cells to identify synthetic combinations of subunits whose pharmacology parallels that of *bona fide* $GABA_A$ receptors *in vivo* (Pritchett *et al.*, *Science*, 1989, 245, 1389; Malherbe *et al.*, *J. Neurosci.*, 1990, 10, 2330; Pritchett and Seeberg, *J. Neurochem.*, 1990, 54, 1802; and Luddens *et al.*, *Nature (London)*, 1990, 346, 648). This approach has revealed that, in addition to an α and β subunit, either γ_1 or γ_2 (Pritchett *et al.*, *Nature (London)*, 1989, 338, 582; Ymer *et al.*, *EMBO J.*, 1990, 9, 3261; and Malherbe *et al.*, *J. Neurosci.*, 1990, 10, 2330) or γ_3 (Herb *et al.*, *Proc. Natl. Acad. Sci. USA*, 1992, 89, 1433; Knoflach *et al.*, *FEBS Lett.*, 1991, 293, 191; and Wilson-Shaw *et al.*, *FEBS Lett.*, 1991, 284, 211) is also generally required to confer benzodiazepine sensitivity, and that the benzodiazepine pharmacology of the expressed receptor is largely dependent on the identity of the α and γ subunits present. Receptors containing a δ subunit (i.e. $\alpha\beta\delta$) do not appear to bind benzodiazepines (Shivers *et al.*, *Neuron*, 1989, 3, 327). Combinations of subunits have been identified which exhibit the pharmacological profile of a BZ_1 type receptor ($\alpha_1\beta_1\gamma_2$) and a BZ_2 type receptor ($\alpha_2\beta_1\gamma_2$ or $\alpha_3\beta_1\gamma_2$, Pritchett *et al.*, *Nature (London)*, 1989, 338, 582), as well as two $GABA_A$ receptors with a novel pharmacology, $\alpha_5\beta_2\gamma_2$ (Pritchett and Seeberg, *J. Neurochem.*, 1990, 54, 1802) and $\alpha_6\beta_2\gamma_2$ (Luddens *et al.*, *Nature (London)*, 1990, 346, 648). Although the pharmacology of these expressed receptors appears similar to that of those identified in brain tissue by radioligand binding, it has nonetheless not been shown that these receptor subunit combinations exist *in vivo*.

A combination of subunits comprising the human $\gamma 3$ $GABA_A$ receptor subunit has not hitherto been possible due to the non-availability of the human $\gamma 3$ cDNA. This has consequently limited the use of cell lines in screening for subtype-specific medicaments, it being impossible to study the pharmacological profile of subunit combinations comprising the $\gamma 3$ subunit.

We have now ascertained the cDNA sequence of the $\gamma 3$ subunit of the human $GABA_A$ receptor. This nucleotide sequence, together with the deduced amino acid sequence corresponding thereto, is depicted in Figure 2 of the accompanying drawings.

The present invention accordingly provides, in a first aspect, a DNA molecule encoding the $\gamma 3$ subunit of the human $GABA_A$ receptor comprising all or a portion of the sequence depicted in Figure 2, or a modified human sequence.

The sequencing of the novel cDNA molecule in accordance with the invention can conveniently be carried out by the standard procedure described in accompanying Example 1; or may be accomplished by alternative molecular cloning techniques which are well known in the art, such as those described by Maniatis *et al.* in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, New York, 2nd edition, 1989.

In another aspect, the invention provides a recombinant expression vector comprising the nucleotide sequence of the $GABA_A$ receptor $\gamma 3$ subunit together with additional sequences capable of directing the synthesis of the said $GABA_A$ receptor $\gamma 3$ subunit in cultures of stably co-transfected eukaryotic cells.

The term "expression vectors" as used herein refers to DNA sequences that are required for the transcription of cloned copies of recombinant DNA sequences or genes and the translation of their mRNAs

in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, blue-green algae, yeast cells, insect cells, plant cells and animal cells. Specifically designed vectors allow the shuttling of DNA between bacteria-yeast, bacteria-plant or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

The term "cloning vector" as used herein refers to a DNA molecule, usually a small plasmid or bacteriophage DNA capable of self-replication in a host organism, and used to introduce a fragment of foreign DNA into a host cell. The foreign DNA combined with the vector DNA constitutes a recombinant DNA molecule which is derived from recombinant technology. Cloning vectors may include plasmids, bacteriophages, viruses and cosmids.

The recombinant expression vector in accordance with the invention may be prepared by inserting the nucleotide sequence of the $GABA_A$ $\gamma3$ subunit into a suitable precursor expression vector (hereinafter referred to as the "precursor vector") using conventional recombinant DNA methodology known from the art. The precursor vector may be obtained commercially, or constructed by standard techniques from known expression vectors. The precursor vector suitably contains a selection marker, typically an antibiotic resistance gene, such as the neomycin or ampicillin resistance gene. The precursor vector preferably contains a neomycin resistance gene, adjacent the SV40 early splicing and

polyadenylation region; an ampicillin resistance gene; and an origin of replication, e.g. pBR322 ori. The vector also preferably contains an inducible promoter, such as MMTV-LTR (inducible with dexamethasone) or metallothionin (inducible with zinc), so that transcription can be controlled in the cell line of this invention. This reduces or avoids any problem of toxicity in the cells because of the chloride channel intrinsic to the $GABA_A$ receptor.

One suitable precursor vector is pMAMneo, available from Clontech Laboratories Inc. (Lee *et al.*, *Nature*, 1981, 294, 228; and Sardet *et al.*, *Cell*, 1989, 56, 271). Alternatively the precursor vector pMSGneo can be constructed from the vectors pMSG and pSV2neo.

The recombinant expression vector of the present invention is then produced by cloning the $GABA_A$ receptor γ_3 subunit cDNA into the above precursor vector. The receptor subunit cDNA is subcloned from the vector in which it is harboured, and ligated into a restriction enzyme site, e.g. the HindIII site, in the polylinker of the precursor vector, for example pMAMneo or pMSGneo, by standard cloning methodology known from the art, and in particular by techniques analogous to those described herein. Before this subcloning, it is often advantageous, in order to improve expression, to modify the end of the γ_3 subunit cDNA with additional 5' untranslated sequences, for example by modifying the 5' end of the γ_3 subunit DNA by addition of 5' untranslated region sequences from the α_1 subunit DNA.

One suitable expression vector of the present invention is illustrated in Fig. 1 of the accompanying drawings, in which R represents the nucleotide sequence of the γ_3 subunit of the $GABA_A$ receptor, and the remainder of the expression vector depicted therein is derived from the precursor vector pMSGneo.

According to a further aspect of the present invention, there is provided a stably co-transfected eukaryotic cell line capable of expressing a $GABA_A$ receptor, which receptor comprises at least one alpha, one beta and the $\gamma 3$ subunit.

This is achieved by co-transfecting cells with three expression vectors, each harbouring cDNAs encoding for an α, β or $\gamma 3$ $GABA_A$ receptor subunit. In a further aspect, therefore, the present invention provides a process for the preparation of a eukaryotic cell line capable of expressing a $GABA_A$ receptor, which comprises stably co-transfecting a eukaryotic host cell with at least three expression vectors, one such vector harbouring the cDNA sequence encoding for an alpha, another such vector harbouring the cDNA sequence encoding for a beta, and a third such vector harbouring the cDNA sequence encoding for the $\gamma 3$ $GABA_A$ receptor subunit. The stable cell-line which is established expresses an $\alpha\beta\gamma 3$ $GABA_A$ receptor. Each receptor thereby expressed, comprising a unique combination of α, β and $\gamma 3$ subunits, will be referred to hereinafter as a $GABA_A$ receptor "subunit combination". Pharmacological and electrophysiological data confirm that the recombinant $\alpha\beta\gamma 3$ receptor expressed by the cells of the present invention has the properties expected of a native $GABA_A$ receptor.

Expression of the $GABA_A$ receptor may be accomplished by a variety of different promoter-expression systems in a variety of different host cells. The eukaryotic host cells suitably include yeast, insect and mammalian cells. Preferably the eukaryotic cells which can provide the host for the expression of the receptor are mammalian cells. Suitable host cells include rodent fibroblast lines, for example mouse Ltk^-, Chinese hamster ovary (CHO) and baby hamster kidney (BHK); HeLa; and HEK293 cells. It is necessary to incorporate at least one α, one β and the

γ_3 subunit into the cell line in order to produce the required receptor. Within this limitation, the choice of receptor subunit combination is made according to the type of activity or selectivity which is being screened for. For example, benzodiazepines (designated BZ) represent one class of drugs which act upon the $GABA_A$ receptor. The presence of an α_1 subunit is specific for a class of benzodiazepines having the pharmacology designated BZ_1; whereas α_2 to α_5 define different pharmacological profiles, broadly designated as BZ_2. The type of β subunit is not critical in defining the class of benzodiazepine, although a β subunit is required. The γ_3 subunit is also important in defining BZ selectivity. It is likely that differentiation between α subunit selectivity is conferred by the γ_3 subunit.

In order to employ this invention most effectively for screening purposes, it is preferable to build up a library of cell lines, each with a different combination of subunits. Typically a library of 5 or 6 cell line types is convenient for this purpose. Preferred subunit combinations include: $\alpha_2\beta_1\gamma_3$ and $\alpha_3\beta_1\gamma_3$, and in particular $\alpha_5\beta_3\gamma_3$. These may be used with cell lines containing other subunit combinations such as $\alpha_1\beta_1\gamma_2$; $\alpha_1\beta_2\gamma_2$; $\alpha_2\beta_1\gamma_1$; $\alpha_2\beta_1\gamma_2$; $\alpha_3\beta_1\gamma_2$; $\alpha_4\beta_1\gamma_2$; $\alpha_5\beta_1\gamma_2$; $\alpha_6\beta_1\gamma_2$; and $\alpha_1\beta_1\gamma_{2L}$.

As stated above, for each cell line of the present invention, three such vectors will be necessary, one containing an α subunit, one containing a β subunit, and the third containing the γ_3 subunit.

Cells are then co-transfected with the desired combination of three expression vectors. There are several commonly used techniques for transfection of eukaryotic cells *in vitro*. Calcium phosphate precipitation of DNA is most commonly used (Bachetti *et al.*, *Proc. Natl. Acad. Sci. USA*, 1977, 74, 1590-1594; Maitland *et al.*, *Cell*, 1977, 14, 133-141), and represents a favoured technique in the context of the present invention.

A small percentage of the host cells takes up the recombinant DNA. In a small percentage of those, the DNA will integrate into the host cell chromosome. Because the neomycin resistance gene will have been incorporated into these host cells, they can be selected by isolating the individual clones which will grow in the presence of neomycin. Each such clone is then tested to identify those which will produce the receptor. This is achieved by inducing the production, for example with dexamethasone, and then detecting the presence of receptor by means of radioligand binding.

In a further aspect, the present invention provides protein preparations of $GABA_A$ receptor subunit combinations, especially human $GABA_A$ receptor subunit combinations, comprising the human γ_3 $GABA_A$ receptor subunit derived from cultures of stably transfected eukaryotic cells. The invention also provides preparations of membranes containing subunit combinations of the $GABA_A$ receptor, especially human $GABA_A$ receptor subunit combinations, comprising the human γ_3 $GABA_A$ receptor subunit derived from cultures of stably transfected eukaryotic cells. In an especially preferred embodiment, the invention provides cell membranes containing a human $GABA_A$ receptor consisting of an $\alpha\beta\gamma_3$ subunit combination isolated from stably transfected mouse Ltk$^-$ fibroblast cells, most especially an $\alpha_5\beta_3\gamma_3$ subunit combination.

The cell line, and the membrane preparations therefrom, according to the present invention have utility in screening and design of drugs which act upon the $GABA_A$ receptor, for example benzodiazepines, barbiturates, β-carbolines and neurosteroids. The present invention accordingly provides the use of the cell line described above, and membrane preparations derived therefrom, in screening for and designing medicaments which act upon the $GABA_A$ receptor. Of particular interest in this context are molecules capable of interacting selectively with

$GABA_A$ receptors made up of varying subunit combinations. As will be readily apparent, the cell line in accordance with the present invention, and the membrane preparations derived therefrom, provide ideal systems for the study of structure, pharmacology and function of the various $GABA_A$ receptor subtypes.

The following non-limiting Examples illustrate the present invention.

EXAMPLE 1

ISOLATION AND SEQUENCING OF cDNAS ENCODING THE HUMAN $GABA_A$ RECEPTOR γ_3 SUBUNIT

a) cDNA libraries

cDNAs were cloned from human foetal brain cDNA libraries. All cDNA libraries were constructed in the lambdaZAP vector, and were purchased from Stratagene (San Diego, California). For screening, the cDNA libraries were plated according to the manufacturer's instructions, at 40,000 pfu per 137 mm plate. Filter lifts were taken using Hybond N filters (Amersham) according to the manufacturer's instructions.

b) Isolation of cDNA encoding human γ_3 subunit

A rat γ_3 cDNA probe was first generated by PCR using oligonucleotide primers derived from the rat γ_3 sequence (Knoflach *et al*, *FEBS Lett.*, 1991, 293, 191):
5'ATTCAAGCTTACCATGGCTGCAAAGCTGCTGCTTCTCTGCCTGTTCT
CGGG3' (bp 177-217, with 13 bases on the 5' end containing a Hind III restriction site) SEQ. ID. NO.: 1, and

5'GGAATTGTTTAACGTGATCATCACGGGTG3' (bp 1330-1358, antisense) SEQ. ID. NO.: 2. PCR was performed as described, for example, by Whiting *et al* in *Proc. Natl. Acad. Sci. USA*, 1990, 87, 9966, using rat brain cDNA as a template. A 1250bp PCR product was obtained which when digested with Hind III was cut into 2 pieces of 900bp and 350bp in size. The 900bp fragment was subcloned into the Hind III site of pBluescript SK-(Stratagene) and its identity confirmed by DNA sequencing using standard techniques and the Sequensase II enzyme (United States Biochemicals).

A human foetal brain cDNA library was screened using ^{32}P labelled rat γ_3 900bp DNA as described above. A single cDNA clone was obtained. Sequence analysis was performed, using an Applied Biosystems 373A DNA sequencer and dye terminator chemistry according to the manufacturers' instructions. This cDNA lacked both the 5' and 3' ends of the coding region. These were subsequently obtained by anchored PCR. For the 3' end, a sense oligonucleotide derived from sequence near the 3' end of the truncated cDNA clone (5'CCAGATTCCTCAAGATGATTCCTGAGCGAATAAG3', incorporating an EcoRI site) SEQ. ID. NO.: 3 was used in conjunction with an oligonucleotide "anchor" primer derived from the T7 primer sequence of the pBluescript vector (5'AGCGCGCGTAATACGACTCACTATAGGGCGAA3') SEQ. ID. NO.: 4 in a PCR reaction with human foetal brain cDNA library as template. A 500bp PCR product was obtained and subcloned into EcoR1 cut pBluescript SK-. Sequencing, as above, confirmed that it contained the 3' end of the human γ_3 coding region, together with 131bp of 3' untranslated region sequence. The missing 5' sequences of the γ_3 cDNA were obtained using human brain "5' RACE Ready cDNA", obtained from CLONTECH (part no. 7302-1), using the antisense primers 5'GCTTTTTATCATATGCTCTTAGCAAC3' SEQ. ID. NO.: 5 and

5'CAAGACCCACATATGGTTTGATGGAGA3' SEQ. ID. NO.: 6, derived from the very 5' end of the truncated γ_3 cDNA clone. The anchored PCR was performed according to manufacturers' instructions, and a 200bp PCR product obtained which was subcloned into the p-CR-Script vector (Stratagene), again according to the manufacturers' instructions. DNA sequencing confirmed that the 200bp PCR product contained the missing 5' coding region of the human γ_3 cDNA, together with 25bp of 5' untranslated region.

The complete nucleotide sequence of the cDNA encoding the human γ_3 subunit, together with the deduced amino acid sequence corresponding thereto is shown in Fig. 2 of the accompanying drawings SEQ. ID. NO.: 7.

EXAMPLE 2

PREPARATION OF STABLY TRANSFECTED CELLS EXPRESSING $\alpha_5\beta_3\gamma_3$ SUBUNIT COMBINATION OF THE HUMAN GABA$_A$ RECEPTOR

Human α_5 (see International patent specification no. WO 92/22652), β_3 (Wagstaff et al, *Genomics*, 1991, 11, 1071) and γ_3 cDNAs were subcloned into the eukaryotic expression vector pMSGneo (the preparation of which is described in WO 92/22652) using standard techniques (cf. Maniatis *et al.*, in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, New York, 2nd Edition, 1989) and a stable cell line expressing human $\alpha_5\beta_3\gamma_3$ GABA-A receptor established according to the methodology described in Example 1 of WO 92/22652.

EXAMPLE 3

CHARACTERISATION OF STABLY TRANSFECTED CELLS EXPRESSING $\alpha_5\beta_3\gamma_3$ SUBUNIT COMBINATION OF THE HUMAN GABA$_A$ RECEPTOR

Expression of recombinant $\alpha_5\beta_3\gamma_3$ human GABA$_A$ receptors is demonstrated by radiological binding. Transfected cells which had been induced by culture in dexamethasone containing medium for 3-5 days (according to methodology described in Example 2 of WO 92/22652) were harvested and cell membranes prepared (again according to methodology described in Example 2 of WO 92/22652). Saturation binding curves (Figure 3) were obtained by incubating cell membranes with various concentrations of ^{3}H Ro15-1788 (obtained from New England Nuclear, Du Pont (U.K.) Ltd., Stevenage), with non-specific binding measured in the presence of 10μM flunitrazepam (obtained from Sigma Chemical Company, Poole, UK). All binding assays were performed in triplicate in an assay volume of 0.5ml, with an incubation time of 90min at 4°C. Incubations were terminated by filtration through GF/B filters (Brandel, Gathersberg, MD) on a Tomtech cell harvester, followed by three washes in ice-cold assay buffer. After drying, filter-retained radioactivity was measured by liquid scintillation counting.

A cell line prepared as described in Example 2 expressed approximately 80fmol [^{3}H]Ro15-1788 binding sites/mg protein following a 5-day induction of receptor expression. The expression of human α_5, β_3 and γ_3 mRNA transcripts was confirmed by isolation of mRNA, cDNA synthesis and PCR using subunit specific oligonucleotide primers in a conventional manner.

Scatchard analysis of saturation binding curves for [^{3}H]Ro15-1788 was performed for membrane preparations from two cell lines expressing the $\alpha_5\beta_3\gamma_3$ subunit combination according to the present invention, giving the following K_D values (mean ± SEM): 0.32±0.06nM and 0.63±0.11nM.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Merck Sharp & Dohme Limited
(B) STREET: Terlings Park
(C) CITY: Harlow
(D) STATE: Essex
(E) COUNTRY: England
(F) POSTAL CODE (ZIP): CM20 2QR

(ii) TITLE OF INVENTION: Nucleic Acids

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTCAAGCTT ACCATGGCTG CAAAGCTGCT GCTTCTCTGC CTGTTCTCGG G 51

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAATTGTTT AACGTGATCA TCACGGGTG 29

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAGATTCCT CAAGATGATT CCTGAGCGAA TAAG 34

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCGCGCGTA ATACGACTCA CTATAGGGCG AA 32

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTTTTTATC ATATGCTCTT AGCAAC 26

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CAAGACCCAC ATATGGTTTG ATGGAGA 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1565 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 33..1436

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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TGAATTCGTG AGATGGCGAG CTCCACGGCA CC ATG GCC CCG AAG CTG CTG CTC      53
                                    Met Ala Pro Lys Leu Leu Leu
                                     1               5

CTC CTC TGC CTG TTC TCG GGC TTG CAC GCG CGG TCC AGA AAG GTG GAA     101
Leu Leu Cys Leu Phe Ser Gly Leu His Ala Arg Ser Arg Lys Val Glu
        10                  15                  20

GAG GAT GAA TAT GAA GAT TCA TCA TCA AAC CAA AAG TGG GTC TTG GCT     149
Glu Asp Glu Tyr Glu Asp Ser Ser Ser Asn Gln Lys Trp Val Leu Ala
    25                  30                  35

CCA AAA TCC CAA GAC ACC GAC GTG ACT CTT ATT CTC AAC AAG TTG CTA     197
Pro Lys Ser Gln Asp Thr Asp Val Thr Leu Ile Leu Asn Lys Leu Leu
40                  45                  50                  55

AGA GAG TAT GAT AAA AAG CTG AGG CCA GAT ATT GGA ATA AAA CCG ACC     245
Arg Glu Tyr Asp Lys Lys Leu Arg Pro Asp Ile Gly Ile Lys Pro Thr
                60                  65                  70

GTA ATT GAC GTT GAC ATT TAT GTT AAC AGC ATT GGT CCT GTG TCA TCA     293
Val Ile Asp Val Asp Ile Tyr Val Asn Ser Ile Gly Pro Val Ser Ser
            75                  80                  85

ATA AAC ATG GAA TAC CAA ATT GAC ATA TTT TTT GCT CAG ACC TGG ACA     341
Ile Asn Met Glu Tyr Gln Ile Asp Ile Phe Phe Ala Gln Thr Trp Thr
        90                  95                  100

GAT AGT CGC CTT CGA TTC AAC AGC ACA ATG AAA ATT CTT ACT CTG AAC     389
Asp Ser Arg Leu Arg Phe Asn Ser Thr Met Lys Ile Leu Thr Leu Asn
    105                 110                 115

AGC AAC ATG GTG GGG TTA ATC TGG ATC CCA GAC ACC ATC TTC CGC AAT     437
Ser Asn Met Val Gly Leu Ile Trp Ile Pro Asp Thr Ile Phe Arg Asn
120                 125                 130                 135

TCT AAA ACC GCA GAG GCT CAC TGG ATC ACC ACA CCC AAT CAG CTC CTC     485
Ser Lys Thr Ala Glu Ala His Trp Ile Thr Thr Pro Asn Gln Leu Leu
                140                 145                 150

CGG ATT TGG AAT GAC GGG AAA ATC CTT TAC ACT TTG AGG CTC ACC ATC     533
Arg Ile Trp Asn Asp Gly Lys Ile Leu Tyr Thr Leu Arg Leu Thr Ile
            155                 160                 165
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AAT GCT GAG TGC CAG CTG CAG CTG CAC AAC TTC CCC ATG GAC GAA CAC 581
Asn Ala Glu Cys Gln Leu Gln Leu His Asn Phe Pro Met Asp Glu His
170 175 180

TCC TGC CCG CTG ATT TTC TCC AGC TAT GGC TAT CCC AAA GAA GAA ATG 629
Ser Cys Pro Leu Ile Phe Ser Ser Tyr Gly Tyr Pro Lys Glu Glu Met
185 190 195

ATT TAT AGA TGG AGA AAA AAT TCA GTG GAG GCA GCT GAC CAG AAA TCA 677
Ile Tyr Arg Trp Arg Lys Asn Ser Val Glu Ala Ala Asp Gln Lys Ser
200 205 210 215

TGG CGG CTT TAT CAG TTT GAC TTC ATG GGC CTC AGA AAC ACC ACA GAA 725
Trp Arg Leu Tyr Gln Phe Asp Phe Met Gly Leu Arg Asn Thr Thr Glu
220 225 230

ATC GTG ACA ACG TCT GCA GGT GAT TAT GTT GTC ATG ACT ATA TAT TTT 773
Ile Val Thr Thr Ser Ala Gly Asp Tyr Val Val Met Thr Ile Tyr Phe
235 240 245

GAA TTG AGT AGA AGA ATG GGA TAC TTC ACC ATT CAG ACA TAC ATT CCC 821
Glu Leu Ser Arg Arg Met Gly Tyr Phe Thr Ile Gln Thr Tyr Ile Pro
250 255 260

TGT ATA CTG ACT GTG GTT TTA TCC TGG GTG TCA TTT TGG ATC AAA AAA 869
Cys Ile Leu Thr Val Val Leu Ser Trp Val Ser Phe Trp Ile Lys Lys
265 270 275

GAT GCT ACG CCA GCA AGA ACA GCA TTA GGC ATC ACC ACG GTG CTG ACC 917
Asp Ala Thr Pro Ala Arg Thr Ala Leu Gly Ile Thr Thr Val Leu Thr
280 285 290 295

ATG ACC ACC CTG AGC ACC ATC GCC AGG AAG TCC TTG CCA CGC GTG TCC 965
Met Thr Thr Leu Ser Thr Ile Ala Arg Lys Ser Leu Pro Arg Val Ser
300 305 310

TAC GTG ACC GCC ATG GAC CTT TTT GTG ACT GTG TGC TTC CTG TTT GTC 1013
Tyr Val Thr Ala Met Asp Leu Phe Val Thr Val Cys Phe Leu Phe Val
315 320 325

TTC GCC GCG CTG ATG GAG TAT GCC ACC CTC AAC TAC TAT TCC AGC TGT 1061
Phe Ala Ala Leu Met Glu Tyr Ala Thr Leu Asn Tyr Tyr Ser Ser Cys
330 335 340

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AGA AAA CCA ACC ACC ACG AAA AAG ACA ACA TCG TTA CTA CAT CCA GAT      1109
Arg Lys Pro Thr Thr Thr Lys Lys Thr Thr Ser Leu Leu His Pro Asp
    345                 350                 355

TCC TCA AGA TGG ATT CCT GAG CGA ATA AGC CTA CAA GCC CCT TCC AAC      1157
Ser Ser Arg Trp Ile Pro Glu Arg Ile Ser Leu Gln Ala Pro Ser Asn
360                 365                 370                 375

TAT TCC CTC CTG GAC ATG AGG CCA CCA CCA CCT GCG ATG ATC ACT TTA      1205
Tyr Ser Leu Leu Asp Met Arg Pro Pro Pro Pro Ala Met Ile Thr Leu
                380                 385                 390

AAC AAT TCC GTT TAC TGG CAG GAA TTT GAA GAT ACC TGT GTC TAT GAG      1253
Asn Asn Ser Val Tyr Trp Gln Glu Phe Glu Asp Thr Cys Val Tyr Glu
            395                 400                 405

TGT CTG GAT GGC AAA GAC TGT CAG AGC TTC TTC TGC TGC TAT GAA GAA      1301
Cys Leu Asp Gly Lys Asp Cys Gln Ser Phe Phe Cys Cys Tyr Glu Glu
        410                 415                 420

TGT AAA TCA GGA TCC TGG AGG AAA GGG CGT ATT CAC ATA GAC ATC TTG      1349
Cys Lys Ser Gly Ser Trp Arg Lys Gly Arg Ile His Ile Asp Ile Leu
    425                 430                 435

GAG CTG GAC TCG TAC TCC CGG GTC TTT TTC CCC ACG TCC TTC CTG CTC      1397
Glu Leu Asp Ser Tyr Ser Arg Val Phe Phe Pro Thr Ser Phe Leu Leu
440                 445                 450                 455

TTT AAC CTG GTC TAC TGG GTT GGA TAC CTG TAT CTC TAAGTGTTGC           1443
Phe Asn Leu Val Tyr Trp Val Gly Tyr Leu Tyr Leu
                460                 465

TCAGAGTGAA GAGTGAAGAG CATTTGGTAC ACACTTGACC TTCTGTCGTC CCCAGACCAG    1503

TAGTGACCAA TCGGGAGTAG CAAGGAAGGA CACTGCCCAG TGTATCTTGT TATAAATGAC    1563

CG                                                                   1565
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 467 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Met Ala Pro Lys Leu Leu Leu Leu Leu Cys Leu Phe Ser Gly Leu His
  1               5                  10                  15

Ala Arg Ser Arg Lys Val Glu Glu Asp Glu Tyr Glu Asp Ser Ser Ser
             20                  25                  30

Asn Gln Lys Trp Val Leu Ala Pro Lys Ser Gln Asp Thr Asp Val Thr
         35                  40                  45

Leu Ile Leu Asn Lys Leu Leu Arg Glu Tyr Asp Lys Lys Leu Arg Pro
     50                  55                  60

Asp Ile Gly Ile Lys Pro Thr Val Ile Asp Val Asp Ile Tyr Val Asn
 65                  70                  75                  80

Ser Ile Gly Pro Val Ser Ser Ile Asn Met Glu Tyr Gln Ile Asp Ile
                 85                  90                  95

Phe Phe Ala Gln Thr Trp Thr Asp Ser Arg Leu Arg Phe Asn Ser Thr
            100                 105                 110

Met Lys Ile Leu Thr Leu Asn Ser Asn Met Val Gly Leu Ile Trp Ile
        115                 120                 125

Pro Asp Thr Ile Phe Arg Asn Ser Lys Thr Ala Glu Ala His Trp Ile
    130                 135                 140

Thr Thr Pro Asn Gln Leu Leu Arg Ile Trp Asn Asp Gly Lys Ile Leu
145                 150                 155                 160

Tyr Thr Leu Arg Leu Thr Ile Asn Ala Glu Cys Gln Leu Gln Leu His
                165                 170                 175

Asn Phe Pro Met Asp Glu His Ser Cys Pro Leu Ile Phe Ser Ser Tyr
            180                 185                 190
```

Gly Tyr Pro Lys Glu Glu Met Ile Tyr Arg Trp Arg Lys Asn Ser Val
195 200 205

Glu Ala Ala Asp Gln Lys Ser Trp Arg Leu Tyr Gln Phe Asp Phe Met
210 215 220

Gly Leu Arg Asn Thr Thr Glu Ile Val Thr Thr Ser Ala Gly Asp Tyr
225 230 235 240

Val Val Met Thr Ile Tyr Phe Glu Leu Ser Arg Arg Met Gly Tyr Phe
245 250 255

Thr Ile Gln Thr Tyr Ile Pro Cys Ile Leu Thr Val Val Leu Ser Trp
260 265 270

Val Ser Phe Trp Ile Lys Lys Asp Ala Thr Pro Ala Arg Thr Ala Leu
275 280 285

Gly Ile Thr Thr Val Leu Thr Met Thr Thr Leu Ser Thr Ile Ala Arg
290 295 300

Lys Ser Leu Pro Arg Val Ser Tyr Val Thr Ala Met Asp Leu Phe Val
305 310 315 320

Thr Val Cys Phe Leu Phe Val Phe Ala Ala Leu Met Glu Tyr Ala Thr
325 330 335

Leu Asn Tyr Tyr Ser Ser Cys Arg Lys Pro Thr Thr Thr Lys Lys Thr
340 345 350

Thr Ser Leu Leu His Pro Asp Ser Ser Arg Trp Ile Pro Glu Arg Ile
355 360 365

Ser Leu Gln Ala Pro Ser Asn Tyr Ser Leu Leu Asp Met Arg Pro Pro
370 375 380

Pro Pro Ala Met Ile Thr Leu Asn Asn Ser Val Tyr Trp Gln Glu Phe
385 390 395 400

Glu Asp Thr Cys Val Tyr Glu Cys Leu Asp Gly Lys Asp Cys Gln Ser
405 410 415

Phe Phe Cys Cys Tyr Glu Glu Cys Lys Ser Gly Ser Trp Arg Lys Gly
420 425 430

Arg Ile His Ile Asp Ile Leu Glu Leu Asp Ser Tyr Ser Arg Val Phe
435 440 445

Phe Pro Thr Ser Phe Leu Leu Phe Asn Leu Val Tyr Trp Val Gly Tyr
450 455 460

Leu Tyr Leu
465

CLAIMS:

1. A stably co-transfected eukaryotic cell line capable of expressing a human $GABA_A$ receptor, which receptor comprises at least one alpha receptor subunit, at least one beta receptor subunit and the gamma-3 receptor subunit.

2. A cell line as claimed in claim 1 wherein the cell line is a rodent fibroblast cell line.

3. A process for the preparation of a eukaryotic cell line capable of expressing a human $GABA_A$ receptor, which comprises stably co-transfecting a rodent fibroblast host cell with at least three expression vectors, one such vector harbouring the human cDNA sequence encoding an alpha receptor subunit, another such vector harbouring the human cDNA sequence encoding a beta receptor subunit, and a third such vector harbouring the human cDNA sequence encoding the gamma-3 $GABA_A$ receptor subunit.

4. A process as claimed in claim 3 wherein the eukaryotic cell line is a rodent fibroblast cell line.

5. A DNA molecule encoding the $\gamma 3$ subunit of the human $GABA_A$ receptor comprising all or a portion of the sequence depicted in Figure 2 herein SEQ. ID. NO.: 7.

6. A recombinant expression vector comprising the nucleotide sequence of the human $\gamma 3$ $GABA_A$ receptor subunit together with additional sequences capable of directing the synthesis of the said human

$\gamma 3$ $GABA_A$ receptor subunit in cultures of stably co-transfected eukaryotic cells.

7. A protein preparation of human $GABA_A$ receptor subunit combinations comprising the human $\gamma 3$ $GABA_A$ receptor subunit derived from a culture of stably co-transfected eukaryotic cells.

8. A membrane preparation containing $GABA_A$ receptor subunit combinations comprising the human $\gamma 3$ $GABA_A$ receptor subunit derived from a culture of stably co-transfected eukaryotic cells.

9. A preparation as claimed in claim 7 wherein the subunit combination derived is the $\alpha 5\beta 3\gamma 3$ subunit combination of the human $GABA_A$ receptor.

10. A preparation as claimed in claim 8 wherein the subunit combination derived is the $\alpha 5\beta 3\gamma 3$ subunit combination of the human $GABA_A$ receptor.

11. The use of the cell line as claimed in claim 1, and membrane preparations derived therefrom, in screening for and designing medicaments which act upon the human $GABA_A$ receptor.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/00834

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12N5/10 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 22652 (MERCK SHARP & DOHME LTD) 23 December 1992 cited in the application see page 5, line 6 - page 5, line 21 see page 11, line 9 - page 11, line 13 see claims 1-16 ---	1-11
Y	FEBS LETTERS, vol. 293, no. 1,2, November 1991 AMSTERDAM NL, pages 191-194, KNOFLACH F;RHYNER T;VILLA M;KELLENBERGER S;DRESCHER U;MALHERBE P;SIGEL E;MOHLER H 'The gamma 3-subunit of the GABAA-receptor confers sensitivity to benzodiazepine receptor ligands' see the whole document ---	1-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1995	15.09.95

Name and mailing address of the ISA	Authorized officer
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International Application No
PCT/GB 95/00834

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, February 1992 WASHINGTON US, pages 1433-1437, HERB A;WISDEN W;LUDDENS H;PUIA G;VICINI S;SEEBURG PH; 'The third gamma subunit of the gamma-aminobutyric acid type A receptor family.' see the whole document -----	1-11

1

Intern al Application No
PCT/GB 95/00834

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9222652	23-12-92	AU-A- 1921192	12-01-93
		CA-A- 2109193	12-12-92
		EP-A- 0589930	06-04-94
		JP-T- 6508023	14-09-94